

**Abstracts of the presentations selected for 2006  
JSAR Outstanding Presentation Award**

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## **Isolation of Various Types of Testicular Germ Cells from *pvasa-Gfp* Rainbow Trout by *Gfp*-Dependent Flow Cytometry**

**Ayaka YANO<sup>1</sup>, Kensuke SUZUKI<sup>1</sup> and Goro YOSHIZAKI<sup>1,2</sup>**

<sup>1</sup>Tokyo University of Marine Science and Technology, <sup>2</sup>SORST, JST

**ABSTRACT.** The separated populations of spermatogenic cells would be a powerful tool for studying molecular events in fish spermatogenesis. In this study, we develop a new method to identify and purify various stages of testicular germ cells from rainbow trout possessing the green fluorescent protein (*Gfp*) gene driven by trout vasa regulatory regions (*pvasa-Gfp* trout). This transgenic trout has testicular germ cells whose *Gfp* fluorescence decreases when spermatogenesis proceeds. Because of this unique characteristic, flow cytometry (FCM) could be a powerful method to isolate different stages of spermatogenic cells from *pvasa-Gfp* trout by use of *Gfp*-intensity as an indicator. To date, however in fish species, there are not enough molecular markers to identify different types of testicular germ cells. Therefore, we first characterized the stage-specific molecular markers to identify isolated germ cells objectively in fish. We cloned rainbow trout homologues of *mili*, *scp3* and *shippo1* as candidate markers for spermatogonia, spermatocyte, and spermatids, respectively. The isolated genes were named as *Rtili*, *Rt-scp3*, and *Rt-shippo1*. Results of *in situ* hybridization clearly proved that *Rtili*, *Rt-scp3*, and *Rt-shippo1* can be used as spermatogonia, spermatocyte, and spermatid markers, respectively. We then isolated testicular cells from *pvasa-Gfp* trout by FCM and determined cell types using above-mentioned molecular markers. First, the testicular cells were fractionated into six fractions (A to E) by FCM based on *Gfp*-intensities. After fractionation, RNA was extracted from the isolated cells and used for real time PCR using the molecular markers. The cell fractions showing strong *Gfp* (A and B) expressed *Rtili*, those showing weak *Gfp* expression (C and D) expressed *Rt-scp3*, and the *Gfp*-negative fraction, E expressed *Rt-shippo1*. These results indicated that the isolated fraction A and B contains spermatogonia, fraction C and D contains spermatocyte, and fraction E contains spermatids. We also classified spermatogonia into two fractions; undifferentiated spermatogonia which contain spermatogonial stem cells (SSCs) and differentiated spermatogonia which do not retain SSC ability. Since none of molecular markers can distinguish the two types of spermatogonia, we further classified spermatogonia by transplantation assay. The assay showed that only strong *Gfp*-positive cells (fraction A) could colonize the recipient gonads, though the weaker *Gfp*-positive cells (fraction B) did not. Because only SSCs can colonize recipient gonads, this result indicates that fraction A mainly contains undifferentiated spermatogonia which contain SSCs and fraction B contains differentiated spermatogonia. Thus, we have established a system to identify and isolate various stages of testicular cells from rainbow trout by combination of *Gfp*-dependent FCM and transplantation assay.

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## **GSDF, A Novel TGF- $\beta$ superfamily Member Expressed in Gonadal Somatic Cells, Enhances Proliferation of Primordial Germ cell and Spermatogonia in Rainbow Trout**

**Etsuko SAWATARI<sup>1</sup>, Shinya SHIKINA<sup>1</sup>, Toshio TAKEUCHI<sup>1</sup> and Goro YOSHIZAKI<sup>1,2</sup>**

*<sup>1</sup>Department of Marine Biosciences, Tokyo University of Marine Science and Technology, <sup>2</sup>SORST, JST*

**ABSTRACT.** Our understanding of the molecular mechanisms of primordial germ cell (PGC) proliferation in fish is quite rudimentary, but it is expected to be controlled by the surrounding somatic cells. We assumed that growth factors that are specifically involved in PGC proliferation are expressed predominantly in the genital ridge somatic cells surrounding PGCs. In order to isolate these growth factors, we made a cDNA subtractive library using cDNA from the genital ridges of 40-days post fertilization rainbow trout embryos as the tester, and cDNA from embryos without genital ridges as the driver. This approach identified a novel cytokine designated Gonadal Soma-Derived Growth Factor (GSDF). The phylogenetic tree analysis suggested that GSDF was a distant and novel member of the TGF- $\beta$  superfamily. Furthermore, GSDF is a unique gene that exists only in teleosts. *In situ* hybridization and immunohistochemistry revealed that GSDF was expressed in genital ridge somatic cells surrounding PGCs during embryogenesis. In ovary and testis, the expression of GSDF was restricted to granulosa cells and Sertoli cells, respectively. To analyze the effect of GSDF on PGC development during early embryogenesis, we performed gene-knockdown experiment using antisense oligonucleotide (AO). The inhibition of GSDF translation resulted in a decrease in the number of PGCs. Furthermore, ectopic PGCs and apoptotic PGCs were not found in AO-injected embryos. These results provide clear evidence that GSDF controls the PGC number in trout embryos. Moreover, to reveal the function of GSDF in the testis, we performed an *in vitro* culture of isolated type-A spermatogonia with recombinant GSDF. The recombinant GSDF showed the proliferation enhancing effect on type-A spermatogonia. Furthermore, this effect was completely blocked by antiserum against GSDF. These results denote that GSDF, a novel member of the TGF- $\beta$  superfamily, plays an important role for proliferation of PGC and spermatogonia.

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## Physiological Role of Metastin in Timing Puberty in Female Rats

**Kenji TAKASE<sup>1</sup>, Yoshihisa UENOYAMA<sup>1</sup>, Junya HIRATA<sup>1</sup>, Shunji YAMADA<sup>1</sup>,  
Hisanori MATSUI<sup>2</sup>, Tetsuya OHTAKI<sup>2</sup>, Hirokazu MATSUMOTO<sup>2</sup>,  
Hiroko TSUKAMURA<sup>1</sup> and Kei-ichiro MAEDA<sup>1</sup>**

*<sup>1</sup>Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601,  
Japan, <sup>2</sup>Takeda Pharmaceutical Co., Ltd., Ibaraki 300-4293, Japan*

**ABSTRACT.** Genome-mapping studies revealed that mutations in the gene encoding GPR54 are associated with lack of puberty onset and hypogonadotropic hypogonadism in human and mouse. Metastin, also called kisspeptin and encoded by a metastasis suppressor gene, KiSS-1, was found as an endogenous ligand for GPR54, and potently stimulates GnRH secretion. These findings imply that metastin-GPR54 signaling is involved in timing the onset of puberty via stimulation of pulsatile GnRH secretion. The present study aimed to determine the changes in metastin expression at mRNA and peptide levels during peripubertal period and whether immunoneutralization of endogenous metastin in the brain blocks the onset of puberty. The arcuate nucleus-median eminence region (ARC-ME), anteroventral periventricular nucleus (AVPV) and medial preoptic area (mPOA) were obtained from Wistar-Imamichi strain female rats on days 21, 26, 31, 36 and 41 of age. Quantitative RT-PCR analysis demonstrated that KiSS-1 mRNA expression in the ARC-ME and AVPV increased significantly during peripubertal period. KiSS-1 expression in the mPOA was low throughout the sampling period. GPR54 mRNA expression showed a change in the AVPV and mPOA with a significant increase during peripubertal period. GPR54 expression in the ARC-ME did not change throughout the sampling period. Immunohistochemistry showed that no metastin-immunoreactive cells were found at prepubertal period but the immunoreactivities were apparent in the ARC-ME and AVPV of postpubertal rats. The enzyme immunoassay demonstrated that metastin contents increased in the ARC-ME and AVPV during peripubertal period. Female rats received an infusion of anti-rat metastin monoclonal antibody (4.68 mg/ml) or normal mouse IgG (4.1 mg/ml) into the third ventricle during peripubertal period from days 25 to 39 of age. In normal mouse IgG-treated controls, the animals showed the first estrus at  $42.8 \pm 2.2$  day of age (mean  $\pm$  SEM) with body weight of  $134.0 \pm 11.2$  g (mean  $\pm$  SEM). On the other hand, the first estrus of the anti-rat metastin-treated animals was significantly delayed at  $54.7 \pm 2.0$  days of age with body weight of  $173.7 \pm 10.4$  g compared with control group. The age of vaginal opening was also delayed in the anti-metastin-treated animals. Thus, the onset of puberty was prevented by immunoneutralizing central metastin in female rats. These results indicate that both populations of metastin neurons are involved in timing puberty onset. It is speculated that the increase in metastin in the ARC-ME may time pubertal increase in GnRH/LH pulses and that in AVPV may play a role in generating subsequent GnRH/LH surges in female rats.

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**Ovarian Gap Junction and Protein Kinase A Signaling Pathway  
Play Essential Roles during LH-induced Acquisition of Oocyte  
Maturational Competence in the Teleost Ayu (*Plecoglossus  
altivelis*)**

**Yoji YAMAMOTO<sup>1</sup>, Goro YOSHIZAKI<sup>1</sup>, Toshio TAKEUCHI<sup>1</sup>,  
Kiyoshi SOYANO<sup>2</sup> and Reynaldo PATIÑO<sup>3</sup>**

*<sup>1</sup>Department of Aquatic Biosciences, Tokyo University of Marine Science and  
Technology, Tokyo, <sup>2</sup>Institute for East China Sea Research, Nagasaki University,  
Nagasaki, Japan, <sup>3</sup>USGS Texas Cooperative Fish & Wildlife Research Unit, Texas Tech  
University, Lubbock, TX, USA.*

**ABSTRACT.** In teleost fishes, oocyte maturation is regulated by maturation-inducing hormone (MIH) produced by follicle cells. Although the sensitivity of oocytes to MIH (oocyte maturational competence; OMC) is acquired by LH, LH signaling pathway from follicle cells to oocyte during acquisition of OMC is still largely uncertain. Our previous study with ayu (*Plecoglossus altivelis*) revealed that the heterologous gap junction (GJ) exists between the oocyte and granulosa cells (O-GC) and this gap junctional structure is essential for the acquisition of OMC. Therefore, we raised one hypothesis that follicle cells sense the LH signal and send a second messenger to the oocyte through GJ formed between O-GC. Prior to determination of this second messenger, we must draw attention to signal transduction pathway activated by LH during acquisition of OMC. In this study, we tested the effects of various protein kinase (PK) inhibitors or activators on LH-induced acquisition of OMC. Ovarian fragments containing incompetent oocytes (MIH insensitive oocytes) were incubated with LH (human chorionic gonadotropin; 100IU/ml) under the presence of various dose of PKA and PKC inhibitors (H8-Dihydrochloride; 1, 10, 100  $\mu$ M, GF109203X; 0.1, 1, 10  $\mu$ M). Next, the ovarian fragments were incubated with PKA and PKC activators (forskolin; 0.01, 0.1, 1  $\mu$ M, phorbol12-myristate 13-acetate; 0.01, 0.1, 1  $\mu$ M) alone. To assess the effects of these treatments, the ovarian fragments treated with above mentioned reagents were cultured with 17, 20 $\beta$ -dihydroxy-4-pregnen-3-one (MIH of the ayu) and the percentages of oocytes that underwent germinal vesicle break down were scored. Further, the levels of ovarian cAMP, a candidate molecule that is expected to be transferred between O-GC via GJ during acquisition of OMC, were measured by enzyme immunoassay. We found that forskolin was able to induce OMC with the same potency as LH, whereas PKA inhibitor suppressed LH-induced acquisition of OMC in a dose dependent manner. Either protein kinase C activator or inhibitor had no effect on the acquisition of OMC. Finally, the level of ovarian cAMP increased on stimulation of OMC by LH. These results suggest that 1) the stimulation of OMC by LH is mediated by PKA-dependent pathway, and 2) a strong candidate for the factors that is transferred between O-GC via GJ is cAMP.

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## **Culture Conditions for Maintaining Survival and Mitotic Activity of Transplantable Type A Spermatogonia in Rainbow Trout**

**Shinya SHIKINA<sup>1</sup> and Goro YOSHIZAKI<sup>1,2</sup>**

*<sup>1</sup>Department of Marine Biosciences, Tokyo University of Marine Science and Technology, <sup>2</sup>SORST, JST*

**ABSTRACT.** Germ cell transplantation is a powerful tool to study gametogenesis in various species. Our previous study showed that spermatogonia, transplanted into peritoneal cavity of trout hatchlings, colonized recipient gonads and produced fully functional sperm and eggs in synchrony with the germ cells of recipient. If an *in vitro*-culture system that allows spermatogonia to expand would be established, combined with the transplantation technique, it would be a powerful tool in the field of both basic and applied biology. Therefore, final objective of this study is establishment of transplantable cell-line derived from spermatogonia, possessing activity to colonize recipient gonads after transplantation and capacity to differentiate into functional sperm and eggs. To establish a culture system of fish germ cells, we performed an optimization of culture conditions for spermatogonia in rainbow trout. As materials, immature testes that only contain type A spermatogonia were used. Spermatogonial survival and mitotic activity was improved when testicular cells were prepared using collagenase or collagenase + Dispase and cultured in Leibovitz's L-15 medium (pH 7.8) supplemented with 10% fetal bovine serum at 10 C, compared with ordinal culture conditions for salmonid cells. When testicular somatic cells were eliminated from cultures, spermatogonial mitotic activity was promoted. In addition, when bovine insulin, extract of 20 day-post-fertilized trout embryo, and human basic fibroblast growth factor were added to culture medium, the mitotic activity of purified spermatogonia increased to 4-fold, 1.5-fold, and 1.25-fold, respectively, compared with that of growth factor-free condition. The effects of combining these three factors were significantly additive, the mitotic activity increased to nearly 7-fold in 19 days of culture, compared with that of growth factor-free condition. The mitosis enhancing activity was maintained for over 30 days of culture. Furthermore, the spermatogonia cultured for 1 month could colonize and proliferate in recipient gonads when they were transplanted into newly hatched embryo. These results suggest that our optimum culture conditions could allow spermatogonia to mitose and maintain their activity to colonize recipient gonads at least for 1 month *in vitro*. This study would represent the first step for establishing a transplantable spermatogonial cell-line that would be useful for surrogate brood stock technology.

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## **Expression and Localization of Relaxin and Its Receptor LGR7 and Their Developing Changes in Boar Testes**

**SIQIN<sup>1</sup>, Takuya AOSHIMA<sup>1</sup>, Hitomi ITO<sup>1</sup>, Itaru MINAGAWA<sup>1</sup>,  
Tatsuo KAWARASAKI<sup>2</sup> and Tetsuya KOHSAKA<sup>1</sup>**

*<sup>1</sup>Laboratory of Animal Reproduction, Faculty of Agriculture, Shizuoka University,  
Shizuoka 422-8529, <sup>2</sup>Shizuoka Swine and Poultry Experimental Station, Kikugawa 439-  
0037, Japan*

**ABSTRACT.** Relaxin (Rlx) has originally been described as a hormone of pregnancy that is best known for its role in parturition in pigs. Recently, its own distinct receptor named as LGR7 is discovered by screening of human genome, and characterized to express in multiple human tissues including the testis. The present study was to identify the boar testis as a source and target tissue of Rlx by characterizing the expression and cellular location of both ligand and receptor of the Rlx signaling system within the testis. Testes were obtained from Duroc boars at immature (7 weeks after birth), pubertal (18 weeks) and mature (38 weeks) periods. The identification of the cells that express Rlx and LGR7 molecules was performed by RT-PCR analysis from fractionated testicular cells, *in situ* hybridization and immunohistochemistry. Also the developing changes of expression of both Rlx and LGR7 were evaluated by using semiquantitative RT-PCR assay and western blot analysis. The RT-PCR analysis from fractionated testicular cells showed that Rlx transcripts were restricted to the Leydig cell fraction, whereas LGR7 transcripts were detected in Leydig cell and seminiferous tubular cell fractions (Sertoli and Germ cells). *In situ* hybridization and immunohistochemistry provided mutually confirmatory results for the expression in the fractionated cells. When examined the profile of Rlx/LGR7 expression during the testicular development, semiquantitative RT-PCR assay showed that Rlx gene expression increased after puberty, whereas LGR7 gene expression changed a little during pubertal development. In the testis at all developmental stages studied, western blot analysis with anti-Rlx revealed that no immunoreactive band corresponding to 6 kDa Rlx mature form was observed, while 18 kDa band of the size expected for proRlx and 11 kDa band that might be degraded form of proRlx were detected. This is also emphasized by RT-PCR data that prohormone convertase 1/3, which is believed to be involved in posttranslational processing by cleavage of B-chain/C-peptide junction in proRlx, was not expressed in the testis, despite its presence observed in pregnant pig corpora lutea. On the contrary, western blot with anti-LGR7 demonstrated that LGR7 protein was detected as a specific band of 75 kDa in all testes studied. In conclusion, we identified the boar testis as a source and target tissue of Rlx by showing that Rlx is expressed in the Leydig cell and that its own distinct receptor LGR7 is expressed in the Leydig cell and seminiferous epithelial cells, suggesting that boar testicular Rlx may contribute to steroidogenesis and spermatogenesis by autocrine and paracrine manner.